A new laboratory model using bull and boar spermatozoa and fluorescent beads to assess a membrane's occlusive potential

Szellö, M ; Janett, F ; Ewald, C ; Music, M ; Sener, B ; Attin, T ; Schmidlin, P R

Abstract: OBJECTIVES The objective of the present study is to assess the potential of bull and boar spermatozoa and fluorescent beads to be used as a surrogate cell model to determine the cell occlusive potential in vitro using membranes of standardized porosities. MATERIALS AND METHODS A two-chamber model system consisting of upper and lower chambers, which could be separated by membranes, was constructed. Isopore polycarbonate membranes with different standardized pore diameters were used to assess the mobile cellular penetration behavior of spermatozoa or the more passive non-cellular permeability of fluorescent particles (beads) of different diameter and color. In a first experiment, spermatozoa were placed in the lower chamber, whereas semen extender only was placed in the upper chamber. After 10 min of incubation at 37 °C, the sperm number was assessed in the latter. In a second experiment, a bead solution was drawn through resorbable collagen membranes from the upper into the lower chamber by vacuum using a syringe and bead number and size was analyzed by flow cytometry. All experiments were carried out in triplicates. A non-porous polyester membrane was used as negative control to assess the overall tightness of the setup. RESULTS Boar and bull spermatozoa had average cell body lengths and widths of 9 × 5 m and were unable to pass through pores 2 m, whereas they were detectable at pore sizes 3 m. Their number increased with increasing pore diameters, i.e., from minimal concentrations of 0.1 × 10(6)/ml for boar and 0.5 × 10(6)/ml for bull spermatozoa at 3 m to maximal concentrations of 2.1 × 10(6)/ml for boar and 13.1 × 10(6)/ml for bull spermatozoa at 8 m. The fluorescent beads followed the expected pattern of permeability reliably correlating bead and pore diameter. CONCLUSIONS Within the limitations of this laboratory study and the xenogeneic cell surrogate material, the model allows to easily assess cell and particle penetration through porous structures like membranes. We hope to further assess, improve, and validate this model, which we aim to use for the screening of dental membranes after being exposed to different degradation methods. CLINICAL RELEVANCE Convenient and rapid test procedures to evaluate membranes for regenerative procedures are still warranted.

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Abstract

Objectives: To assess the potential of bull and boar spermatozoa and fluorescent beads to be used as a surrogate cell model to determine the cell occlusive potential *in vitro* using membranes of standardized porosities.

Materials and Methods: A two-chamber model system consisting of upper and lower chambers, which could be separated by membranes, was constructed. Isopore polycarbonate membranes with different standardized pore diameters were used to assess the mobile cellular penetration behavior of spermatozoa or the more passive non-cellular permeability of fluorescent particles (beads) of different diameter and color. In a first experiment, spermatozoa were placed in the lower chamber, whereas semen extender only was placed in the upper chamber. After 10 min of incubation at 37°C the sperm number was assessed in the latter. In a second experiment, a bead solution was drawn through resorbable collagen membranes from the upper into the lower chamber by vacuum using a syringe and bead number and size was analyzed by flow cytometry. All experiments were carried out in triplicates. A non-porous polyester membrane was used as negative control to assess the overall tightness of the set-up.

Results: Boar and bull sperms had average cell body lengths and widths of 9x5 µm and were unable to pass through pores ≤ 2 µm, whereas they were detectable at pore sizes ≥ 3µm. Their number increased with increasing pore diameters, i.e. from minimal concentrations of 0.1×10^6/ml for boar and 0.5×10^6/ml for bull spermatozoa at 3 µm to maximal concentrations of 2.1×10^6/ml for boar and 13.1×10^6/ml for bull spermatozoa at 8 µm. The fluorescent beads followed the expected pattern of permeability reliably correlating bead and pore diameter.

Conclusions: Within the limitations of this laboratory study and the xenogeneic cell surrogate material, the model allows to easily assess cell and particle penetration through porous structures like membranes. We hope to further assess, improve and validate this model, which we aim to use for the screening of dental membranes after being exposed to different degradation methods.

Clinical Relevance: Convenient and rapid test procedures to evaluate membranes for regenerative procedures are still warranted.

1. Introduction

Membranes in dentistry are used in several indications and locations for surgically guided regenerative procedures to allow for an undisturbed formation of tissues like bone (guided bone regeneration) or periodontal ligament (guided tissue regeneration) in a protected area separated from fast growing unspecific undesired cell populations like connective tissue or epithelium. Several types of material are available for this purpose, however, main criteria like occlusivity, biocompatibility, spaciousness, clinical manageability and an appropriate healing have to be fulfilled [1]. Resorbable and non-resorbable membranes can be used, but many conventional membranes still possess some structural, mechanical, and biofunctional limitations and the "ideal" membrane for use in regenerative therapy has yet to be developed [2].

As mentioned above, the main prerequisite for successful defect regeneration remains the establishment of protected and separated compartments to allow for guided bone and/or tissue regeneration. As such, the membranes should not allow for rapid invasion and permeation of bacteria and unwanted host cells, while being adequately integrated into the respective tissues, thus allowing for undisturbed healing. The evaluation of cell proliferation on and through membranes is still a challenge in routine laboratory screening of membranes and it is not sufficient to assess the cell proliferation on surfaces but also within the membrane as done so in most laboratory investigations. Since growing cells under laboratory conditions is complex and time-consuming, an easy cell assay would be desirable to assess membrane permeability and occlusive capacity. While the proliferation and differentiation of different cell types of interest on the surface of different membranes has been rather well investigated, the methods for the evaluation of the penetration potential of cells through membranes remains scarce [3-5].

One study assessed explicitly the penetration of prokaryotic cells, namely of *S. mutans* and *A. actinomycetemcomitans* through guided tissue regeneration (GTR) membranes using a device

consisting of an inner tube and an outer bottle filled with culture media. The study showed differences in the behavior of the evaluated membranes and – not surprisingly - that an incorporation of an antibiotic into the membrane was effective in controlling bacterial percolation [6]. According to the authors' knowledge studies using such models are, however, still lacking the evaluation of eukaryotic cells.

Therefore, the aim of the present study was to establish a surrogate model and to design a laboratory device to measure membrane tightness. For this purpose we used xenogeneic spermatozoa from bulls and boars to identify a threshold pore size below which these mobile cells are unable to penetrate and above which these cells can pass, respectively. As a control, we used fluorescent beads representing particles lacking motility and flexibility to gain insight into the correlation between pore size and ability to penetrate a membrane for rigid particles as opposed to motile viable cells.

2. Materials and Methods

2.1. Cell types

Frozen-thawed bull and liquid-preserved boar semen was provided by the Clinic of Reproductive Medicine (Vetsuisse-Faculty, University of Zurich, Switzerland). For preservation and dilution, AndroMed® (Minitube, Tiefenbach, Germany) and Androhep® (Minitube, Tiefenbach, Germany) media were used for bull and boar semen, respectively. To keep the temperature constantly at 37° C, the trial was performed on a heating plate and in an incubation chamber. Sperm concentration was determined using computer-assisted sperm analysis (IVOS II, Hamilton Thorne Biosciences, Beverly MA, US), which also allowed the tracking of spermatozoa trajectories.

2.2 Chamber system

A custom-made two-chamber model made of polyoxymethylene (POM) was fabricated. POM is semi-crystalline, mostly linear and a thermoplast fabricated through chain-polymerisation and chain-copolymerisation, respectively. It is of a high dimensional stability, has an excellent slide and closure capacity, and is biologically inert. Using a regular metal screw, the two chambers can be joined to each other. The upper and lower chamber have a volume of 2 ml each. The chambers can be separated by different test membranes, which were fixed between the chambers by elastic o-rings above and below the membrane (Fig. 1). The chamber floors had additional access holes, which could be closed by rubber plugs to allow liquid application and sampling.

2.3 Membranes and test procedure

The tests were performed with six isopore membrane filters (Merck Millipore, Darmstadt, Germany) of different pore size (0.6, 1.2, 2.0, 3.0, 5.0, 8.0 µm; Fig. 2). Membranes ranged from 7-22 µm in thickness and from 5-20 % porosity, respectively. An impermeable polyester membrane (PR-P 100 MY, folex®, Switzerland) was used as negative control to assess the overall tightness of the set-up.

2.3.1 Spermatozoa experiments

The lower chamber was loaded with extended semen containing 50-200 x 10^6 spermatozoa/ml until the chamber was completely filled. The respective membrane was then carefully placed between two o-rings on top of the lower chamber, and the upper chamber (chamber 2) tightly fixed on top of the first one, thereby having the membrane set in between the two chambers with the o-rings in place. Then, the upper chambers were filled from the top with sperm-free AndroMed® and Androhep® media for bull and boar sperm, respectively, and closed with rubber plugs. The two-chamber system was turned and was then incubated at 37° Celsius for

10 min and a sample taken from the upper collecting chamber, which previously contained no sperm cells and the solution analyzed by phase-contrast microscopy using computer-assisted sperm analysis (IVOS II, Hamilton Thorne Biosciences, Beverly MA, USA). Only descriptive data presentation was provided. All experiments with the two different sperm populations and the different membranes were carried out in triplicates each.

2.3.2. Fluorescent bead experiments

The same type of porous membranes and impermeable foil as negative control was used for the bead experiments. Additionally a resorbable collagen membrane (Resodont forte, Resorba, Nürnberg, Germany) was used to further verify our model system in the context of an exemplary membrane used in oral surgery. All membranes were rinsed in phosphate buffered saline (PBS; #10010001 Gibco, Life Technologies, USA) to remove any contaminating particles before use. The bead mixture contained 1 µm (Dragon Green, Bangs Laboratories Inc., USA), 4 µm and 6 µm beads (7 color set-up beads, #335775 BD Biosciences, USA) in PBS, at a ratio of 1.5:1:1.4 for 1 µm : 4 µm : 6 µm beads, respectively. The 1 µm beads were identified by their light scattering properties and their color to exclude any overlap with background noise originating from small environmental dust particles in the solution, whereas the 4 and 6 µm beads could be identified based on their scatter parameters alone due to their increased size separating their scatter signals from the background. First, the upper chamber was filled with PBS, then the o-rings, membranes and the upper chamber were assembled on top of it as described above. The lower chamber was left empty and used to collect solution after passing through the membranes when a vacuum was applied with a syringe. This was performed once with PBS to rinse membranes and chambers. The resulting solutions were discarded. Then a 2 ml bead solution was introduced into the upper chamber and vacuum was applied to the lower chamber and the solution, which had passed through the membrane was analyzed by flow cytometry on an LSR Fortessa (BD Biosciences, USA) using Diva 8.0.1

software. For the 1 μm beads forward and side scatter were recorded as well as the green fluorescence of the beads excited by a 488 nm laser and detected between 515 and 545 nm. Bead populations of different size were identified based on their light scattering properties. Based on this, bead numbers were determined by counting all events falling into a gate of a specific population. For 1 μm beads only green fluorescent events were counted due to the high level of background noise caused by dust particles of similar size. Again, all experiments were carried out in triplicates.

2.4 Scanning Electron microscopy (SEM)

Surface structure topography of the membranes was examined by a scanning electron microscope at magnifications of 5000x at the Center of Microscopy and Image Analysis, University of Zürich (SEM; Carl Zeiss Supra 50 VP FESEM, Carl Zeiss). For this purpose, the samples were fixed for 24h in 2.5% glutaraldehyde solution. Afterwards, the membranes were rinsed with PBS and dehydrated in ascending concentrations of alcohol (50, 70, 80 und 90%) twice for 15 minutes. Finally, membranes were immersed three times for 15 min in 94% and 60 min in 100% ethanol. Samples were the subjected to critical point drying (Bal-tec CPD030), mounted on SEM mounts (Baltec AG, Blazers, Liechtenstein) and were gold sputtered (Balzers SCD 030, Balzers Union, Balzers, Liechtenstein) for 60 s in an argon gas atmosphere at a target distance of 50 mm, a pressure of 5 Pascal (Pa) at 45 mA. SEM images were taken at a working distance of 9.2 mm and a acceleration voltage of 10 kV.

2.5 Data analysis

The number of sperm cells was assessed by phase-contrast microscopy using computer-assisted sperm analysis. Only a descriptive data presentation was provided.

3. Results

Bull and boar spermatozoa represented comparable cell body dimensions: Widths of approximately 5 µm and lengths of 9 µm could be confirmed microscopically using Olympus analySIS® image software (Olympus, Volketswil, Switzerland) in both cell populations, which were available for our experiments (Fig. 2).

The membrane dimensions as claimed by the manufacturers could also be confirmed by SEM analysis. The number of pores, however, varied between the different membranes. Membranes with larger pore diameters displayed fewer pores due to the otherwise inevitable overlaps of pores.

The qualitative evaluation of sperm cell patency through the membranes showed a clear and distinct pattern: Spermatozoa passage could be found only with pore sizes of ≥ 3 µm, with more sperm cells observed with increasing pore sizes. Upon qualitative inspection, no significant difference was observed between bull and boar sperm cells (Table 1; Fig. 3 and 4).

The quantitative evaluation corroborated these findings and no spermatozoa were detected in membranes with pores ≤ 2 µm. Their number increased with increasing pore diameters, i.e. from minimal concentrations of 0.1 $\times 10^6$/ml for boar and 0.5 $\times 10^6$/ml for bull sperm at 3 µm to maximal concentrations of 2.1 $\times 10^6$/ml for boar and 13.1 $\times 10^6$/ml for bull sperm at 8 µm, respectively. At the intermediate pore size of 5 µm, penetrating sperm concentrations ranging from 0.24 to 0.4 and 0.9 to 2.4 $\times 10^6$/ml for boar and bull sperm were identified, respectively.

Only in the sample of boar number 1 at a pore diameter of 3 µm, no spermatozoa could be counted, while cells were subjectively observed under the microscope. The original concentration did not seem to influence the number of percolating cells.

More bull sperm cells were counted at membrane pore sizes of 3 and 5 µm as compared to the boar samples.

The bead experiments followed a similar pattern of membrane permeability, however, with a much tighter correlation between actual particle size and pore size (Table 2 and Fig.5). Beads

of 1 µm diameter did pass through 1.2 µm pores, but in smaller numbers as compared to membranes with pores of 2 µm found under the conditions described above. For all pore sizes > 2 µm, a roughly four-fold increase in the number of 1µm beads was observed. Beads of 4 µm did not pass through membranes of ≤ 3µm, but comparable numbers of beads were observed for membranes with pore sizes of 5 µm and 8 µm. Beads of 6 µm did not pass through membranes of ≤ 5µm, a significant number of beads passed through membranes with pore size of 8 µm. Figure 6 shows a representative SEM image of a 3 µm membrane after testing with remaining beads on the surface. When a resorbable dental membrane was used, bead solution could only be drawn through the membrane after incubating it in PBS for 24 hours and even after pre-incubation only a small number of beads passed through the membrane. The observed bead numbers were comparable to membranes with pore sizes of 1.2 µm.

4. Discussion

This study was designed to test a novel laboratory model to assess the cell occlusive potential of membranes of different standardized porosities *in vitro*. For this purpose, bull and boar spermatozoa as well as non-motile rigid beads of a known size were used.

As principle finding, this study clearly showed that both cell types under investigation were not able to pass through pores ≤ 2 µm, whereas only pore sizes of ≥ 3 µm revealed detectable spermatozoa in the evaluation chamber. Their number increased with increasing pore diameters despite decreasing porosities.

Animal studies provided clear evidence that cell occlusion and space-provision positively influence the magnitude of alveolar bone regeneration in conjunction with guided tissue regeneration [7]. Polimeni and co-workers created critical-size supra-alveolar periodontal defects in Beagle dogs, which were treated either with space-providing ePTFE devices with or without laser-drilled pores. However, these pores had much larger diameters of 300 µm

compared to the diameters assessed in the present investigation. The actual limit of diameter, which is still tolerable for a successful outcome is a matter of debate and the available literature is scarce. However, there appears to be a tendency for operated sites receiving porous membranes to remain more predictably submerged for primary intention healing, as compared to sites treated with occlusive membranes, which exhibit more wound failures and membrane exposures [8, 9]. In humans, the regenerative response expressed as gain in vertical attachment level or new bone formation has also been found to be better compared to sites when membranes become exposed [10, 11]. It has been suggested that more porous membranes facilitate the blood supply and nutrition of the flaps and the overall biocompatibility of membranes types. An immunohistochemical animal study showed that different patterns of transmembraneous angiogenesis exist among different collagen membranes after subcutaneous implantation in rats [12], which also can be related – in part to some histologically observed structure differences and the degradation behavior of the membranes [13]. Overall, even after two weeks, vascularization may still be incomplete and one may assume that the diffusion of smaller sized cells like blood and endothelial cells to be beneficial, especially at early stages of membrane integration. Erythrocytes for instance display diameters of 3-4 µm. Endothelial cells display widths and lengths of 7-8 µm and 90-144 µm, respectively [14, 15]. Therefore, the sperm cell types under investigation in this study with cell body dimensions ranging from 5-9 µm are in the lower end of physiologically relevant range of cell sizes.

Sperm head dimensions - expressed as their head widths and lengths – may, vary between different species but also between and within breeds of stallions [16], however, they have comparable dimensions in bull and boar as shown in the present study. In addition, comparable penetration patterns through the membranes were evident. The fact that these cells are easily available and allow for fast screening tests within minutes avoiding complex culture techniques make them - in principle - a valuable surrogate for this kind of test. A

noteworthy feature of the chamber model is that it allows for a manipulation of the membranes between different penetration experiments, which would be difficult under growing conditions in culture media. However, one must also take into consideration that spermatozoa are susceptible to even the slightest chemical alterations, which might interfere with their motility and viability and therefore hamper their permissive potential and thus the interpretation of the overall results. Noteworthily, the discrepancy between the actual size of the sperm head and pore size permeability was an intriguing finding and reflects a methodological difference between the selected surrogate models using flexible cells or a rigid particle system such as beads.

With the exception of the bead based experiments, the present feasibility study focused on porous membranes with simple tubular aspects. The resorbable collagen membrane used as a control material in the bead experiments showed a very low permeability for beads of 6 and 4 µm, respectively and only a very moderate permeability for 1 µm beads. Approximately 3% of the total number of 1 µm beads in solution passed the Resodont F membrane, however only after the membrane had been soaked in PBS over night. The permeation behavior of cells might be different in more complex interconnected structures like dental membranes. Whether spermatozoa are able to break through these has yet to be tested. In any case, it would be advisable to combine this cell detection approach with an evaluation of the kinetics of mass transport for a better understanding of nutrition and vascularization phenomena in membranes as well in future studies. This has previously been shown to provide valuable information in biofilm research [17].

In summary and within all the limitations of the present laboratory investigation, the proposed xenogeneic cell surrogate model allows for a rather simple cell penetration evaluation of porous structures like membranes. In contrast to the common indication sites of membranes and the familiar homing cells serving for such models, the cells used in the current study were biochemically completely different from those acting in the regenerative zone, namely white

blood cell lines, fibroblast, epithelial cells, etc. However, since growing these cells under laboratory conditions is complex and time-consuming, the proposed penetration assay using spermatozoa allows for a fast screening of the membrane permeability and the occlusive capacities. It should be noted that despite the obviously advantageous handling characteristics, this model is sensitive to accurate handling technique and relies on the availability of live sperm cells and the support from reproductive medical centers and experts in this field to ensure reliable results.

In the future, we hope to further assess, improve and validate this model, which we aim to use for the screening of dental membranes at different stages of degradation.

Conflict of Interest

The authors declare no conflicts of interest.

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Table 1. Results of the three individually run experiments (No. 1-3) using either bull or boar spermatozoa and the descriptive results of the quantitative and qualitative, evaluation methods.

	Quantitative evaluation*				**Qualitative evaluation**		
Bull sperm	No. 1	No. 2	No.3		No. 1	No. 2	No.3
original conc. (mio/ml)	57.6	193.5	163.2		57.6	193.5	163.2
Control	0	0	0		-	-	-
0.6 µm	0	0	0		-	-	-
1.2 µm	0	0	0		-	-	-
2.0 µm	0	0	0		-	-	-
3.0 µm	0.5	1.9	0.31		+	+	+
5.0 µm	0.9	2.4	1.5		++	++	++
8.0 µm	2.2	4.2	13.1		+++	+++	+++
Boar sperm	No. 1	No. 2	No.3		No. 1	No. 2	No.3
original conc.	94.1	79.8	155.9		94.1	79.8	155.9
Control	0	0	0		-	-	-
0.6 µm	0	0	0		-	-	-
1.2 µm	0	0	0		-	-	-
2.0 µm	0	0	0		-	-	-
3.0 µm	0	0.1	0.1		+	+	+
5.0 µm	0.24	0.4	0.4		++	++	++
8.0 µm	2.08	0.5	1.8		+++	+++	+++

* in Mio

Table 2. Results of three individually run bead experiments. “Bead mix” represents the composition of beads before the experiment. All other values represent bead numbers found in solution after being drawn through a membrane.

Pore diameter in membrane [µm]		Bead mix	0.6 µm	1.2 µm	2 µm	3 µm	5 µm	8 µm	membrane
Average bead number	6 µm	1217.5	0.0	0.3	0.3	1.0	0.7	1378.8	0.6
	4 µm	872.5	0.3	1.1	1.1	2.1	815.1	760.5	5.7
	1 µm	1341.0	0.0	254.3	1151.0	935.6	1021.6	1060.4	36.5

Fig 1. Experimental set-up. Two blocks (A) containing four chambers (a) each were fabricated. The blocks have threaded holes, which allowed for superimposition and fixing of the upper and lower blocks with pins (b) and screws (c). The chamber could be tightly sealed with rubber rings (d) that were placed in the upper and lower chambers individually. Membranes were placed between the blocks individually for each experiment (e/f).

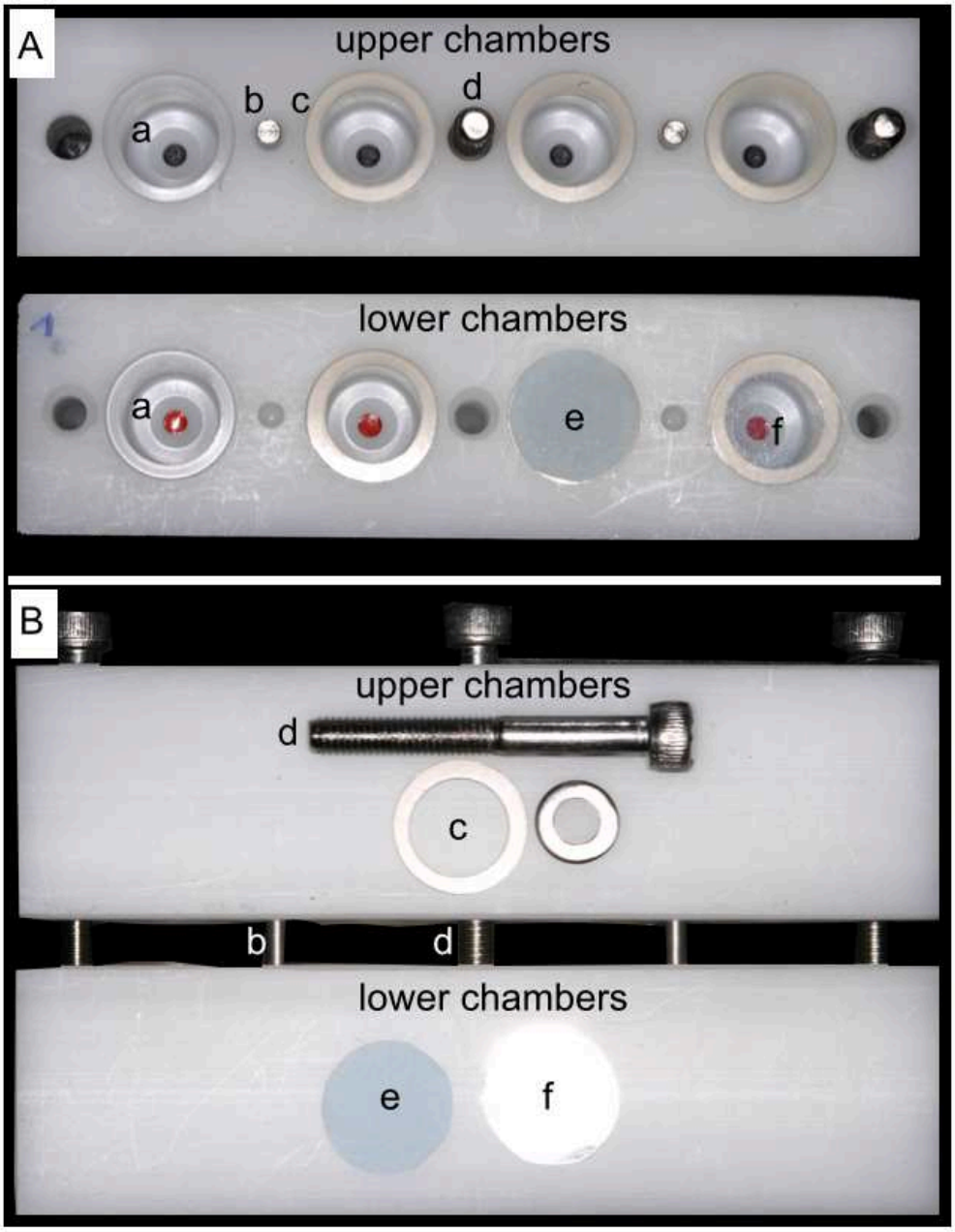

Fig 2. SEM images of the test membranes used in the present study with pore sizes of 0.6 (A), 1.2 (B), 2.0 (C), 3.0 (D), 5.0 (E) and 8.0 μm (F), respectively, taken at a magnification of 5000x.

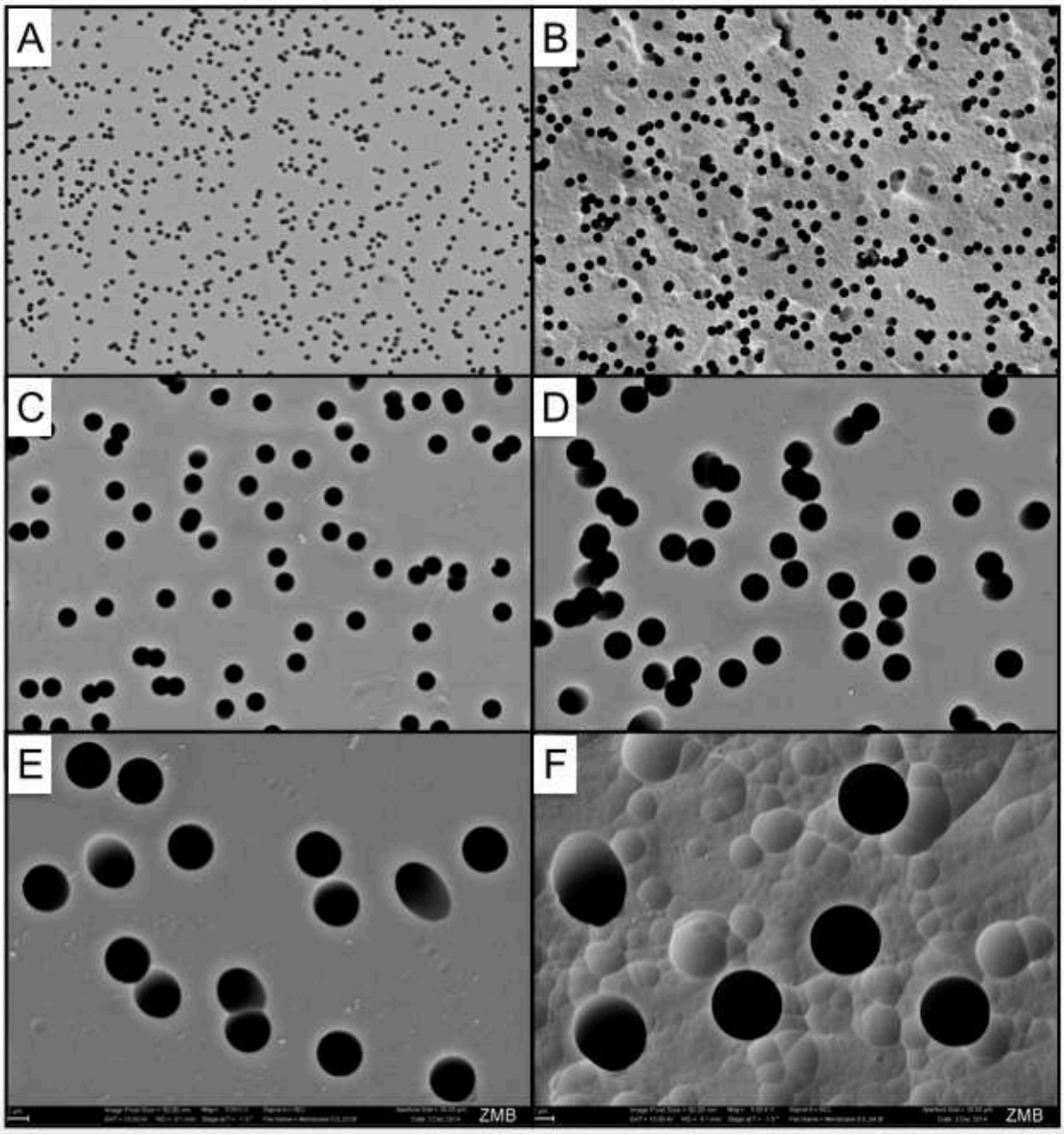

Fig 3. Images of bull and boar spermatozoa visualizing the cell dimensions with comparable average cell body lengths and widths of 9x5 µm in both cell types.

Fig 4. Overview of representative bull and boar sperm images and their spermatozoa tracks.

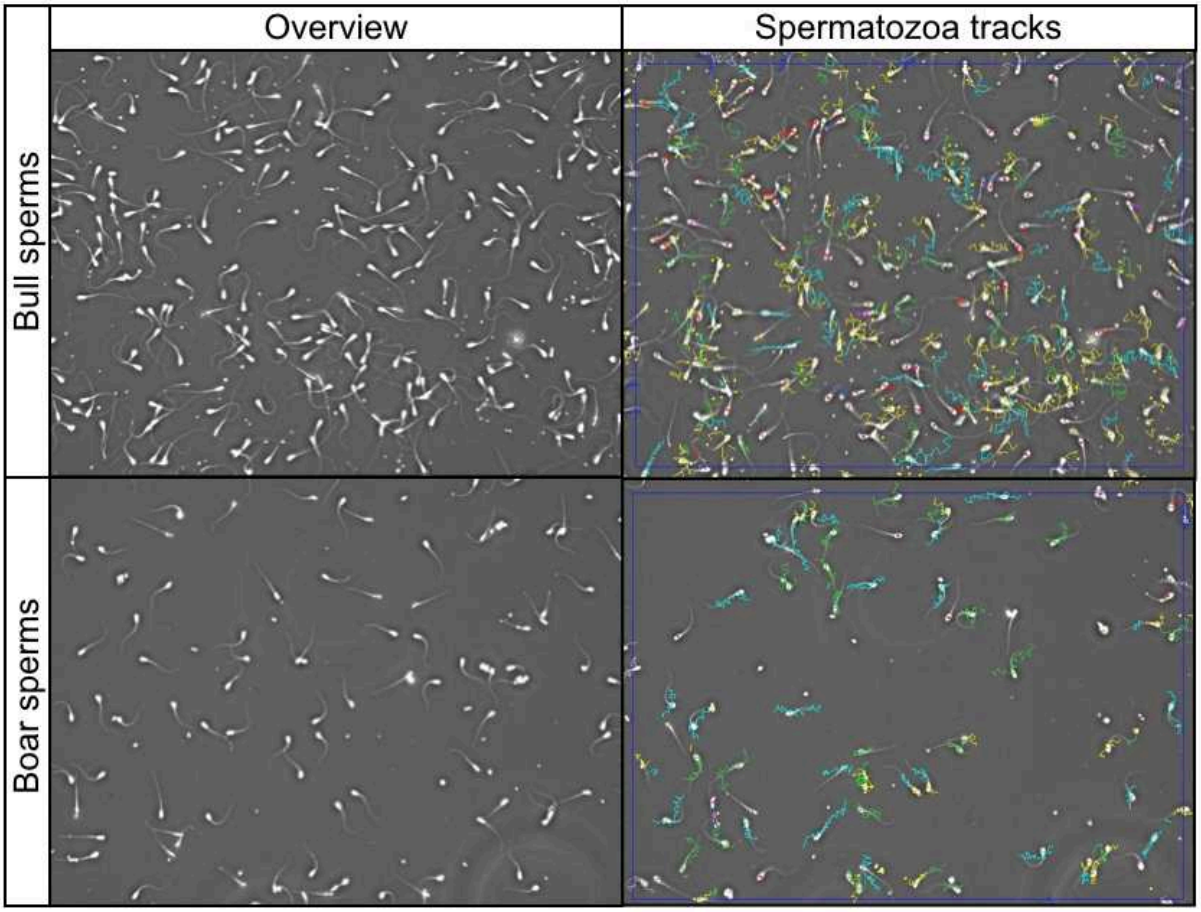

Fig. 5. Representation of experimental values from Table 2. Results of three individually run bead experiments. “Bead mix” represents the composition of beads before the experiment. All other values represent average bead numbers found in solution after being drawn through a membrane.

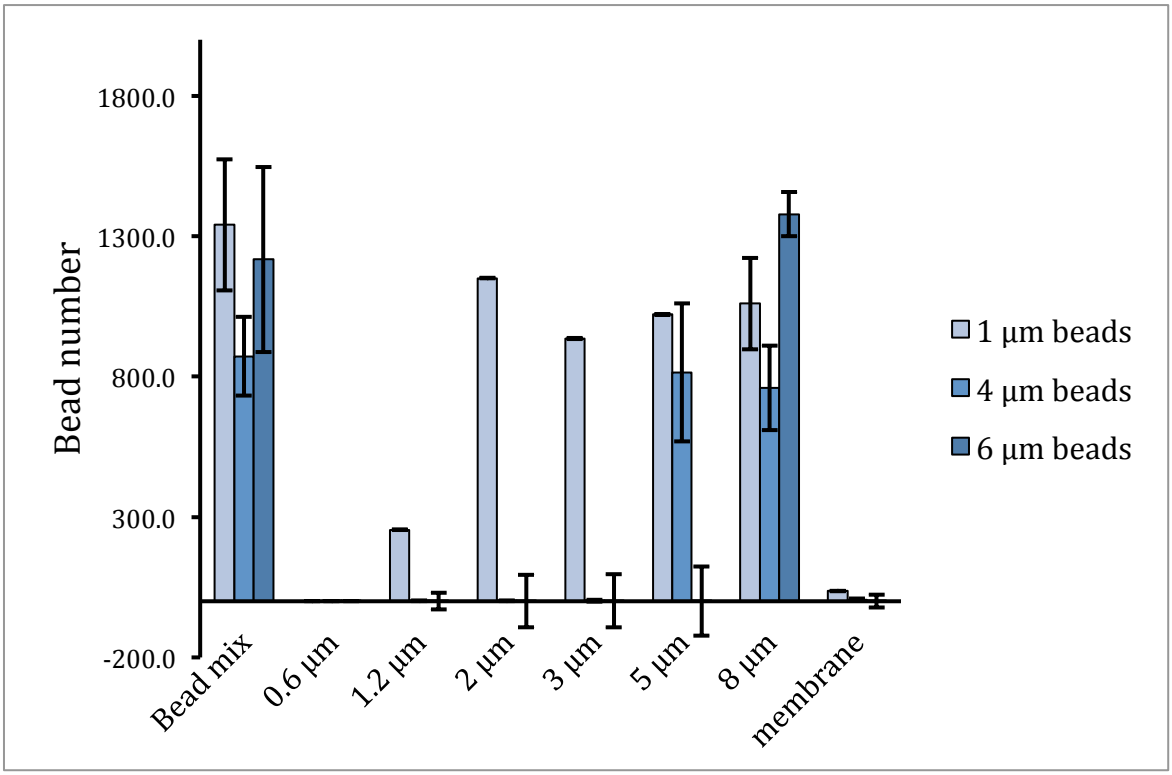

Fig. 6. SEM image of a 3 µm membrane after testing with some beads remaining on the surface (a: 6 µm; b: 4 µm; c: 1 µm). Some 1 µm beads were just entering or penetrating the orifices of the pores (d).The asterisk indicates a 3µm pore.